

## Stabilizers against heat-induced aggregation of RPR 114849, an acidic fibroblast growth factor (aFGF)

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### Abstract

In an effort to optimize stabilization conditions for RPR 114849, a wide variety of known stabilizers were screened for their effects on the stability of the protein against thermal denaturation. For the screening purpose, the effects of excipients on aggregation rate were examined employing UV spectrophotometric turbidity measurements at 50°C and pH 7.4. The protein is sensitive to aggregation near its isoelectric point. Higher concentrations of the protein promote faster aggregation. Reducing agents do not decrease the aggregation rate indicating that oxidation of thiol groups to intermolecular disulfide bonding is not a rate-limiting factor in the aggregation process. In addition to well-known heparin, a wide variety of sulfated and phosphorylated anionic polymers have shown to be powerful stabilizers for the protein. The chain length of a polymeric anion is a critical factor in stabilizing the protein aggregation. The stabilizing effect approaches a constant value asymptotically as the chain length increases. The combined action of enoxaparin and sodium citrate is additive indicating that the stabilizers act independently and do not affect each other's mode, degree, or efficacy of action. High concentrations of non-specific stabilizers, such as sugars and polyols, are capable of suppressing aggregation of the protein to a minor extent. Surfactants, gelling and microencapsulating agents were found to have no practical utility in stabilizing the protein. © 1998 Elsevier Science B.V. All rights reserved.

**Keywords:** Fibroblast growth factor; FGF; Stabilizer; Aggregation; Protein stability

### 1. Introduction

The heparin-binding (fibroblast) growth factor (FGF) family of proteins have potent mitogenic

activity for a variety of target cells (Burgess and Maciag, 1989). RPR 114849, an acidic FGF (aFGF), is a single chain peptide of 154 amino acids with a molecular weight of 17.3 kDa, containing three free thiols. Currently, RPR 114849 is being investigated for a potential clinical indication in the treatment of bone-fracture repair.

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Optimizing stabilization conditions of therapeutic protein formulations is an important practical concern. Many isolated proteins are only marginally stable in solutions due to their propensity to aggregate. To prevent the loss of native conformation, proteins must be protected from environmental conditions causing physical instability as well as chemical decomposition. As protein molecules denature, the hydrophobic domains are often exposed to solvents. The hydrophobic interaction between unfolded proteins results in the formation of non-covalent aggregates.

A study was designed to optimize the stability of RPR 114849 in aqueous solutions in the presence of known pharmaceutical additives. A wide variety of polyanions have been known to stabilize FGF family of proteins from heat-induced inactivation (Kajio et al., 1992; Volkin et al., 1993; Tsai et al., 1993; Chen et al., 1994a; Vemuri et al., 1994). Stabilizers for the protein were screened from known protein stabilizers and heparin-like polyanions with respect to protection from aggregation. Although FGFs are known to be susceptible to chemical degradation such as deamidation of asparagine residues (Shahrokh et al., 1994; Volkin et al., 1995), we focused on the effect of aggregation in limiting the shelf-life. In the present study, turbidity measurements by UV spectroscopy were employed to evaluate the effects of known protein stabilizing agents such as polyanions, polyols, sugars, surfactants, gelling and microencapsulating agents on the heat-induced aggregation of the protein in aqueous solutions. The effects of protein concentration, pH and reducing agents on the aggregation rate were studied to characterize the aggregation process.

## 2. Materials and methods

### 2.1. Materials

All chemicals were either USP grade or of the highest purity available and were used without further purification. Mannitol, sorbitol, dextrose, trehalose, bovine albumin, proline, sarcosine, glycine, *N,N*-dimethylglycine, betaine, benzalkonium chloride, phosvitin, Tween 20, Tween 80,

Triton X-100, chondroitin sulfate C, adenosine triphosphate (ATP), dextran sulfate, heparin sodium, pentosan sulfate, phytic acid, 2-mercaptoethanol, and various types of sodium phosphate glass were purchased from Sigma. Hydroxylpropyl- $\beta$ -cyclodextrin (HPCD) was from American Maize Products. Hydroxylpropyl methyl cellulose (HPMC) and cetyltrimethylammonium bromide (CTAB) were from Aldrich. Sodium dodecyl sulfate (SDS) was from Eastman Kodak. Disodium ethylenediamine tetraacetate (EDTA), sucrose, sodium citrate and sodium sulfate were from Fisher. Dithiothreitol was from Baker. Glycerol was from EM Science. RPR 114849 and enoxaparin were from Rhone-Poulenc Rorer.

### 2.2. Size-exclusion HPLC

The HPLC system consisted of a Perkin Elmer 410 pump, a Perkin Elmer ISS 100 automatic injector, a Perkin Elmer 480 diode array detector. The HPLC method employed a 300  $\times$  7.8 mm I.D. 5- $\mu$ m particle size, silica-based TSK G2000SWXL column (Supelco). Typically, 10  $\mu$ l of a sample (1 mg/ml) was loaded onto the column and eluted with a mobile phase consisting of 20 mM phosphate buffer of pH 6.8 and 0.33 M sodium chloride at a flow rate of 1 ml/min. The detector wavelength was set at 215 nm. Molecular weight was estimated using a standard protein mix (Bio-Rad) containing thyroglobulin (670 kDa),  $\gamma$ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and cyanocobalamin (1.35 kDa).

### 2.3. UV spectroscopy

The rate of aggregation was monitored by UV absorbance increase due to light scattering at 350 nm using a Beckman DU 640 spectrophotometer equipped with 6-cell holder and Peltier temperature programmer. In each experiment, 50  $\mu$ l of 1 mg/ml RPR 114849 stock solution and 450  $\mu$ l of a control phosphate buffer solution (33 mM, pH 7.4 at room temperature) or five solutions containing 33 mM phosphate buffer and graded concentrations of the excipients listed in Table 1 were loaded into a 500- $\mu$ l micro-cuvette and mixed.

The solution was not preheated and incubated at 50°C within the spectrophotometer. It took approximately 2–3 min for the solution to equilibrate with the cell holder temperature. UV absorbance at 350 nm was measured continuously. The turbidity caused by protein aggregation led to an apparent increase in UV-absorbance readings. The absorbance change with increasing temperature was studied at a heating rate of 1 degree/min.

#### 2.4. SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was carried out under reducing (2-mercaptoethanol) and non-reducing conditions using 16% polyacrylamide gels (Novel

Experimental Technology). Samples were prepared and loaded on the plate after heating at 90°C for 3 min. When the electrophoresis was completed, the gels were stained with Coomassie blue or silver stain (Novel) and then destained accordingly.

### 3. Results and discussion

#### 3.1. Aggregation monitoring

UV absorbance versus time for heat-induced aggregation of RPR 114849 in increasing phosphate buffer (pH 7.4) solutions follows the pattern shown in Fig. 1A. The aggregation of the protein does not change in a continuous manner as the temperature is raised. Instead, the protein exhibits little or no change until a point is reached where there is a dramatic change in aggregation behavior. The curve exhibits a lag time, followed by rapid and slow aggregation phases. The same type of curve was obtained in the presence of other excipients. The lag time is partly due to the time taken for the solution to equilibrate with the cell holder temperature. As shown in the figure, increasing buffer concentration inhibits the rate of aggregation. In evaluating the rate of aggregation, the steepest part (dA/min) of a slope of absorbance versus time plot was taken. The instrument was able to calculate slopes at various time points and the highest number was taken. When the solutions were left for a long time, apparent decrease in turbidity was observed due to settlement of large aggregates. The absorbance-temperature profile for the heat-induced aggregation at various concentrations of phosphate buffer is shown in Fig. 2A. Aggregation temperature gradually increases with increasing buffer concentration as the buffer stabilizes the protein.

#### 3.2. Effect of pH

Fig. 3 shows the pH dependence of the aggregation rate at 50°C. The effect of pH was examined in a pH range of 3–8 in citrate, citrate-phosphate (McIlvaine's buffer), phosphate and borate buffers. Since the nature of buffer species and

Table 1  
Stabilizing constants ( $k$ ) in (mg/ml)<sup>-1</sup> for stabilizers in the heat-induced aggregation of RPR 114849 at pH 7.4 and 50°C (The protein concentration was 0.1 mg/ml)

Stabilizers	$k$ (mg/ml) <sup>-1</sup>
NaCl	0.17
Na <sub>2</sub> HPO <sub>4</sub>	0.55
Na <sub>2</sub> SO <sub>4</sub>	0.96
NaF	0.10
Sodium citrate	1.3
Chondroitin C	2.7
Risedronate <sup>®</sup>	7.6
ATP	20
Phosvitin	350
Dextran sulfate	350
Heparin sodium	350
Pentosan sulfate	380
Enoxaparin	420
Phytic acid	450
Sorbitol	0.01
Mannitol	0.008
Glycerol	0.02
Dextrose	0.01
Trehalose	0.007
Sucrose	0.01
Albumin	0.03
Proline	0.01
Sarcosine	0.01
<i>N,N</i> -Dimethylglycine	0.01
Betaine	0.06
SDS	11
HPCD	0.008
HPMC	0.45

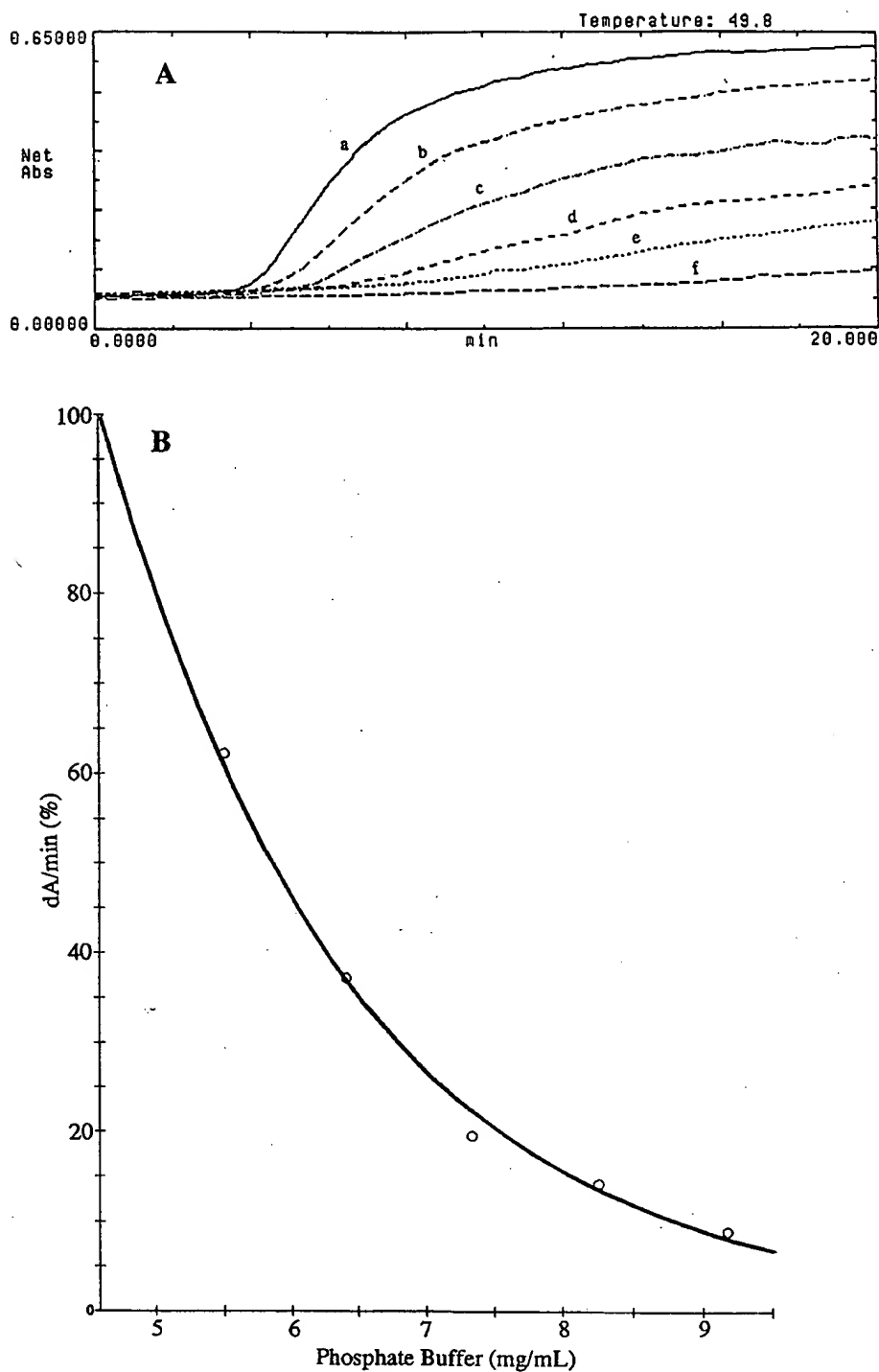


Fig. 1. (A) Time course of heat-induced aggregation of RPR 114849 (0.1 mg/ml) in the presence of various concentrations of phosphate buffer (pH 7.4) at 50°C. Curve [concentration (mg/ml)]: a, 4.59; b, 5.51; c, 6.42; d, 7.34; e, 8.26; f, 9.18. (B) Dependence of the aggregation rate for RPR 114849 (0.1 mg/ml) on phosphate buffer concentration at pH 7.4 and 50°C. The curve drawn through data points is the non-linear regression line fitted with a function,  $y = Ae^{-kx}$ , where  $x$  is the phosphate buffer concentration, and  $y$  is the maximum aggregation rate normalized to the control.

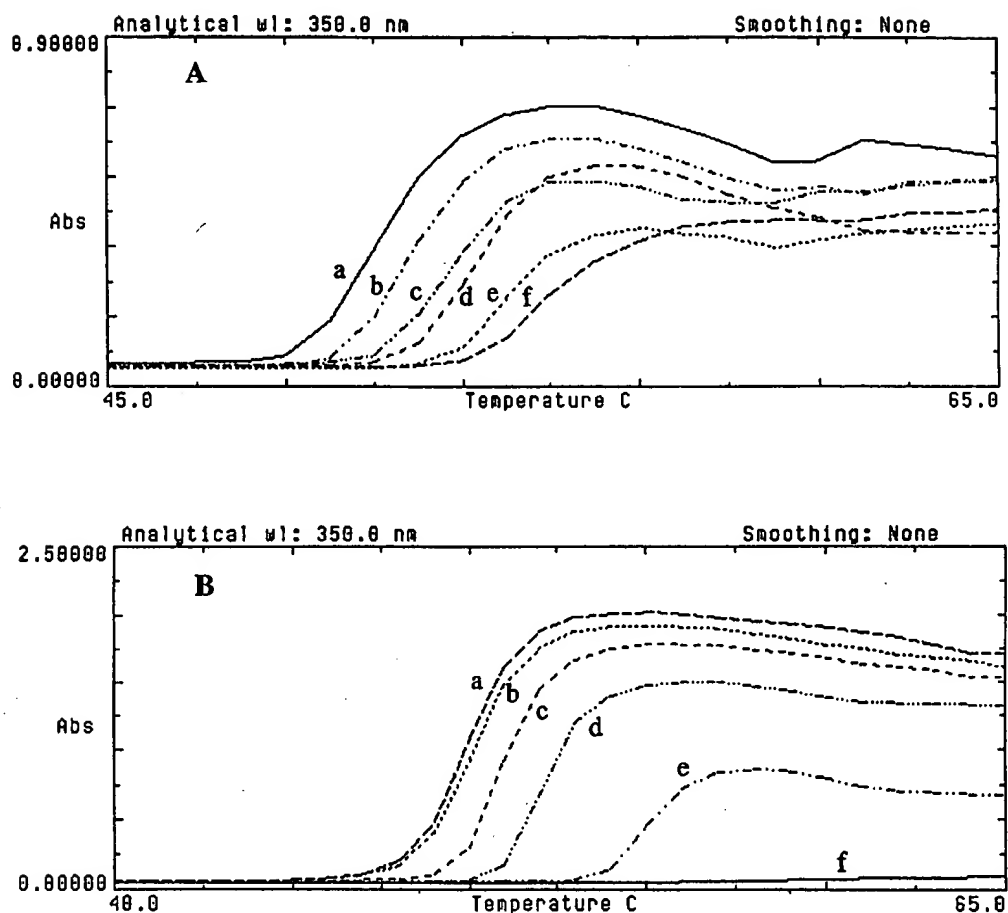


Fig. 2. (A) Effect of increasing phosphate buffer concentration on the aggregation temperature of RPR 114849 (0.1 mg/ml) at pH 7.4. Curve [concentration (mg/ml)]: a, 4.59; b, 5.51; c, 6.42; d, 7.34; e, 8.26; f, 9.18. (B) Effect of the initial concentration of RPR 114849 on the aggregation temperature at pH 7.4. Curve [concentration (mg/ml)]: a, 1.0; b, 0.8; c, 0.6; d, 0.4; e, 0.2; f, 0.1.

buffer concentration play important roles in the stability of proteins, the true pH-dependence of the aggregation is difficult to evaluate. The protein is relatively stable below pH 3.5 and above 7 and appears to become more sensitive to aggregation as the solution pH approaches the isoelectric point ( $pI = 5-6$ ) of aFGFs (Lobb et al., 1986). In general, as the isoelectric point of a protein is reached, the number of neutral molecules increases, which allows hydrophobic forces to predominate. Subtle changes in protein conformation result in the formation of irreversible protein aggregates (Cleland et al., 1993). All the screening experiments were carried out at pH 7.4 to maintain the solution near physiological conditions and maximize the stability.

### 3.3. Effect of protein concentration

The relationship between initial protein concentration and aggregation temperature at pH 7.4 is shown in Fig. 2B. The protein aggregates at lower temperatures with increasing protein concentration. The protein concentration also has a profound effect on the aggregation rate. The dependence of the aggregation rate on the protein concentration exhibited a sigmoidal shape (Fig. 4). Assuming denaturation is a concentration-independent process (Chen et al., 1994b), a wider lag between denaturation and aggregation is observed as the protein concentration lowers. Aggregation results from the denaturation of a protein followed by hydrophobic interactions between un-

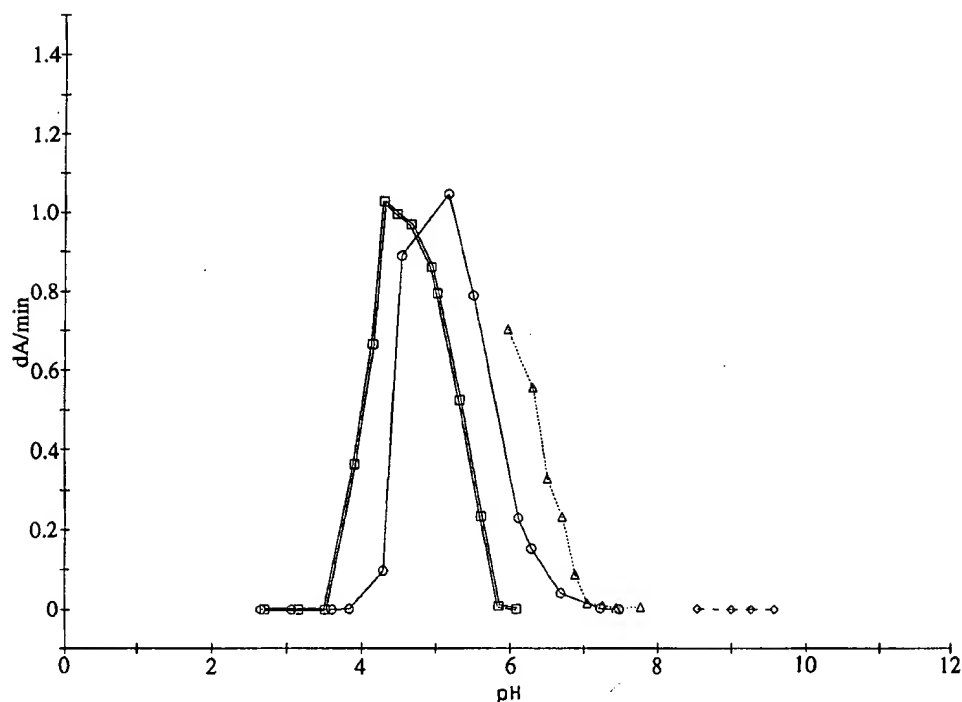


Fig. 3. Effect of pH on the aggregation rate of RPR 114849 (0.1 mg/ml) at 50°C. McIlvaine's citrate-phosphate (○); citrate (□); phosphate (△); borate buffer (◇). Buffer concentration of citrate, phosphate and borate buffers was 67 mM.

folded protein chains. Since aggregation is a multi-molecular process between denatured protein chains, a longer life time for the monomeric denatured protein is expected in dilute solutions. At concentrations higher than 1 mg/ml, the aggregation rate appears to approach a maximum value asymptotically, which may represent denaturation rate of the protein under the conditions. At higher protein concentrations, the monomeric denatured protein chains have a short life-time and associate to form aggregates and hence the denaturation becomes the rate-limiting step in the aggregation process.

### 3.4. Effect of stabilizer concentration

The plot of the steepest slope (dA/min) of the turbidity-versus-time curve for graded concentrations of phosphate buffer in Fig. 1A results in a curve as shown in Fig. 1B. The experimental aggregation rate undergoes simple exponential decrease, as the phosphate buffer concentration increases and the rate approaches zero

asymptotically. The experimental points were fit with an exponential function,  $y = Ae^{-kx}$ , as an empirical model, where  $x$  is the buffer concentration,  $y$  is the normalized aggregation rate (absorbance/min),  $A$  is the normalized aggregation rate for the control, and  $k$  is defined as the aggregation 'stabilizing constant' specific for the buffer. The  $k$  values were obtained from a linear plot of

$$\log y = -kx/2.303 + \log A \quad (1)$$

The  $k$  values on a weight basis obtained for other stabilizers are given in Table 1. The correlation coefficients for the linear fit ranged from 0.95 to 0.99. Due to extremely wide range of molecular weights of the additives, their ability to stabilize the protein was compared on a weight basis rather than a mol basis.

### 3.5. Aggregate characteristics

The heat-induced aggregation was found to be irreversible, as the precipitate could not be solubi-

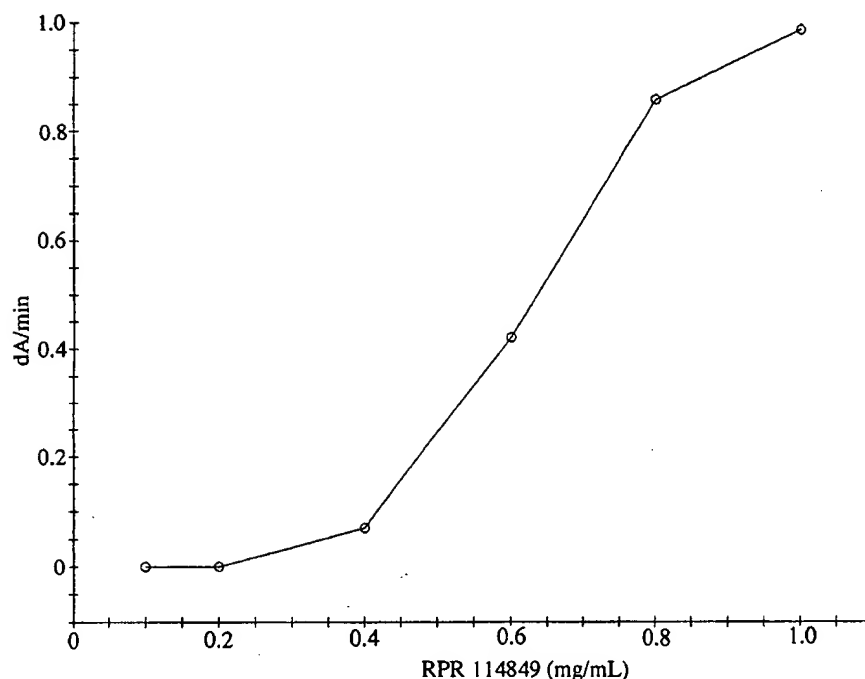


Fig. 4. Effect of the initial concentration of RPR 114849 on the aggregation rate at pH 7.4 and 50°C.

lized upon resuspending in buffer. Only chaotropes (urea and guanidine HCl) were effective. The irreversibility and their solubilization only by chaotropes suggest that the aggregates are hydrophobically associated denatured protein. SDS-PAGE analysis of the chaotrope-solubilized aggregates under reducing and non-reducing conditions shows that a large part of the aggregate is composed of monomers held together by non-covalent forces (Fig. 5). The analysis under non-reducing conditions also revealed dimers, trimers, and other oligomers in addition to the monomers. Almost all high molecular weight bands disappeared under reducing conditions. The aggregate analysis suggests that the aggregation involves, at least in part, autooxidation of thiol groups to disulfide bridges. Since trace metal ions can catalyze sulfhydryl autooxidation, EDTA, a metal chelating agent, was evaluated along with reducing agents (2-mercaptoethanol and dithiothreitol) for their ability to stabilize the protein. However, the reducing agents and EDTA show no stabilizing effect (Fig. 6A). On the contrary, they are destabilizing. This argues that oxidation of thiol groups to interchain disulfide bonds is unlikely to

be the rate-limiting step of the thermal aggregation process.

Only monomeric material was observed when the protein in lag phase and soluble fraction of the aggregated sample were assessed by size-exclusion chromatography, suggesting that the unfolded protein chains do not form soluble dimers or multimers and rather precipitate out of a solution as soon as they associate to form non-covalent or covalent aggregates. Although disulfide-bond formation was found to occur along the aggregation pathway, it doesn't appear to be the rate-limiting step. Conformational instability prior to the disulfide bonding is rather the most likely cause of the thermal aggregation. Therefore, additives that maintain the protein in the native state and prevent the generation of partially or fully unfolded intermediates would be expected to be the most effective stabilizers (Chen, 1992).

### 3.6. Stabilization by anions

A wide diversity of anionic species has been found to stabilize the protein and it appears that

there is low structural specificity. Known FGF-specific stabilizers have common property, i.e. high density of negative charge. The stabilizing effect of the polyanions has been speculated to be due to the electrostatic interaction between the positively charged heparin-binding site and the anion of the ligand, thus decreasing the electrostatic repulsion between positively charged residues of the protein and producing enhanced structural integrity (Volkin et al., 1993; Chen et al., 1994b).

As expected, heparin-like polyanions and sulfated and phosphorylated polymers exhibited pronounced stabilizing effect on the protein, which is consistent with the findings on other FGFs (Kajio et al., 1992; Tsai et al., 1993; Volkin et al., 1993; Chen et al., 1994a; Vemuri et al., 1994). Heparin, dextran sulfate, pentosan sulfate, enoxaparin, phosvitin and phytic acid all show extremely powerful stabilizing effect (Table 1). ATP and chondroitin sulfate C are less effective.

Inorganic longer chain condensed phosphates were also found to be very powerful stabilizers. When the stabilizing constant of the condensed

phosphate was plotted against the chain length of the phosphate, a Langmuir-type curve was obtained (Fig. 7). The monomer, orthophosphate, stabilizes the protein only to a minor extent. As the chain length of the phosphate group is increased, the stabilizing constant increases. The most marked effect appears to occur when the number of the phosphate group is smaller than 5. The stabilizing activity approaches a constant value asymptotically as the chain length increases further. Condensed phosphates of longer chain length may enhance its ability to interact better with the tertiary-structure-induced positive binding sites of the protein (Volkin et al., 1993). Risedronate®, a bisphosphonate having two phosphate groups, also shows good stabilizing effect.

Among the small molecules, phosphate, sulfate, citrate ions were found to be effective, the citrate ion being the most powerful. Citrate ion has already been known to be able to prevent aggregation of other members of FGF family to a great extent (Chen et al., 1994a; Chen and Arakawa, 1996).

### 3.7. Effect of combined action of sodium citrate and enoxaparin

The combined action of two stabilizers should be and are predictable on the basis of the potencies determined from their individual concentration–slope (dA/min) relations as shown in Fig. 1B. Data from the individual concentration–slope relations (curves not shown) indicated that enoxaparin was more than 300 times as active as sodium citrate on a weight basis. From the concentration–slope curves for enoxaparin and sodium citrate, three dA/min levels of 0.025, 0.036 and 0.074 were chosen. The concentration that produced dA/min of 0.025 was 3.7 µg/ml of enoxaparin or 1.2 mg/ml of sodium citrate, dA/min of 0.036 was 2.5 µg/ml of enoxaparin or 0.8 mg/ml of sodium citrate, and dA/min of 0.074 was 0.9 µg/ml of enoxaparin or 0.3 mg/ml of sodium citrate. Mixtures were prepared so as to be equipotent in their combined action at the three levels. The mixtures contained from 0 to 100% enoxaparin with the residual percentage of equipotent sodium citrate. The aggregation rates

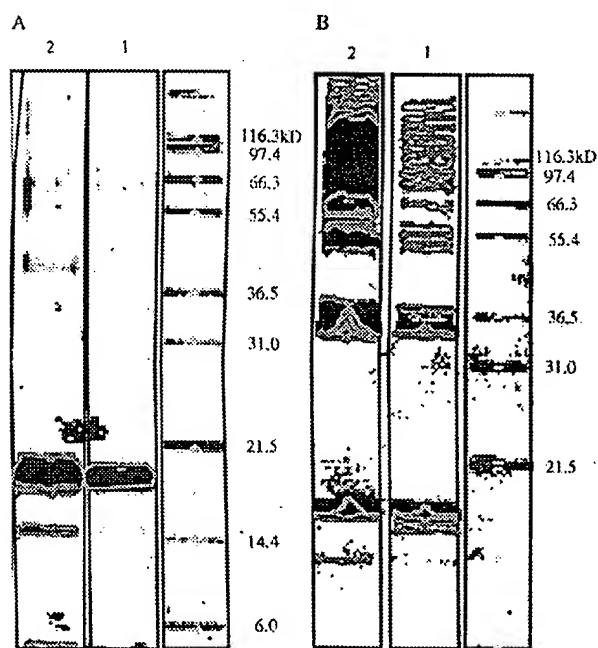


Fig. 5. Silver-stained SDS gels of aggregates under reducing (A) and non-reducing (B) conditions. Lane 1: 250 µg; Lane 2: 625 µg.



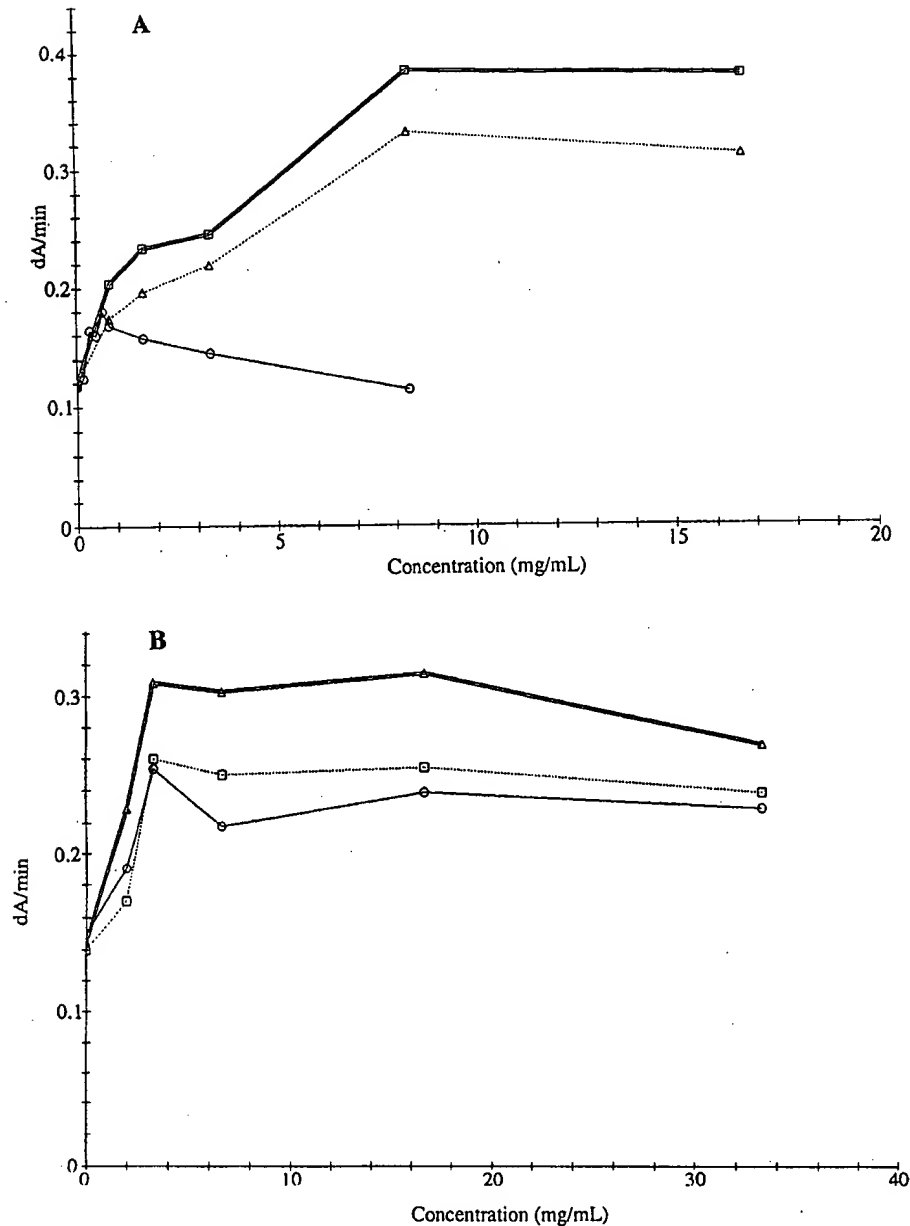


Fig. 6. (A) Aggregation rates of RPR 114849 in phosphate buffer of pH 7.4 at 50°C in the absence and presence of various concentrations of EDTA (○), dithiothreitol (□) and 2-mercaptoethanol (Δ). (B) Effect of Tween 20 (○), Tween 80 (□) and Triton X-100 (Δ) concentrations on the aggregation rate of RPR 114849 at pH 7.4 and 50°C.

affected by the mixtures of enoxaparin and sodium citrate are plotted in Fig. 8. The roughly null slopes of the plots at the three different levels demonstrate equivalence of action and lack of any synergism or antagonism between the two stabilizers.

### 3.8. Stabilization by non-specific entities

Polyhydric alcohols, sugars and certain amino acids have been cited in scientific literature for their ability to improve non-specifically the physical stability of many protein preparations by re-

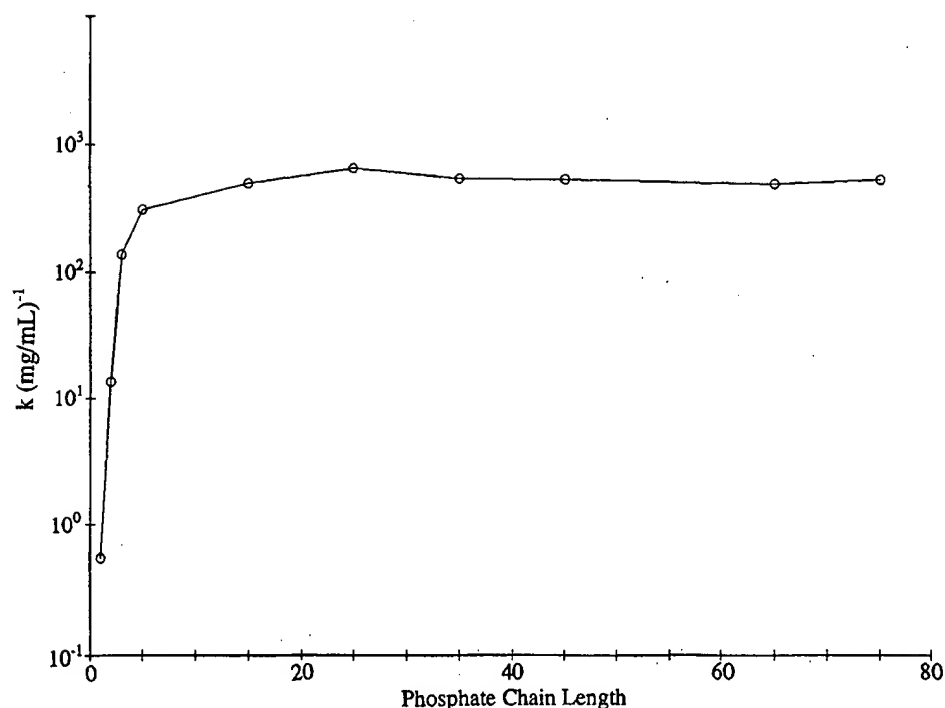


Fig. 7. Effect of chain length of sodium phosphate glass on the inhibitory constant ( $k$ ) in the aggregation of RPR 114849 at pH 7.4 and 50°C.

ducing aggregation (Wang and Hanson, 1988; Manning et al., 1989). These additives are known to stabilize the native state structure through preferential exclusion from the surface of proteins as a result of surface tension (Arakawa et al., 1991; Timasheff, 1992).

The aggregation rate was found to be reduced at sufficiently high concentrations of sucrose, dextrose, trehalose, glycerol, sorbitol, mannitol and proline (Table 1). Glycine has been shown to be effective in inhibiting heat-induced aggregation of some FGFs (Tsai et al., 1993; Chen and Arakawa, 1996). The amino acid, however, was found to be destabilizing at concentrations up to 83 mg/ml of the protein, whereas *N,N*-dimethylglycine is slightly stabilizing (Table 1). Bovine albumin reduces aggregation only at high concentrations, whereas phosvitin, a phosphoprotein, is extremely effective, because of its multiple phosphate groups capable of interacting with cationic binding sites of the protein.

### 3.9. Effect of surfactants

Some surfactants are known for their ability to prevent denaturation and aggregation and are often employed as additives in protein formulations (Loughheed et al., 1983; Chawla et al., 1985). Surfactants are used in protein formulations for their ability to bind to hydrophobic interfaces that are potential denaturation sites, thus reducing aggregation (Cleland et al., 1993). Aggregation rate of RPR 114849 decreases with increasing concentrations of SDS, an anionic surfactant (Table 1). In contrast to SDS, cationic and non-ionic surfactants offered no advantage against aggregation. The aggregation rate increased with increasing concentrations of cationic surfactants (benzalkonium chloride and CTAB) at lower concentrations but the rate decreased at higher concentrations. The non-ionic surfactants (Tween 20, Tween 80 and Triton X-100) accelerated the aggregation rate (Fig. 6B). A commercial dish-washing detergent containing mixed C8 am-

phocarboxylates (Intercon Biodecontamination Group) also showed an effect similar to that of the cationic surfactants.

Non-ionic surfactants may decrease the surface tension and disrupt the hydration layer of proteins, and thus destabilize the native protein structure. Being a well known denaturant, SDS may denature the protein, coat the protein surface and prevent it from aggregation. An alternate interpretation would be that anionic surfactants may stabilize the protein by directly binding with the positively charged binding site and thus providing conformational stability like many of the FGF-specific anionic stabilizers described above.

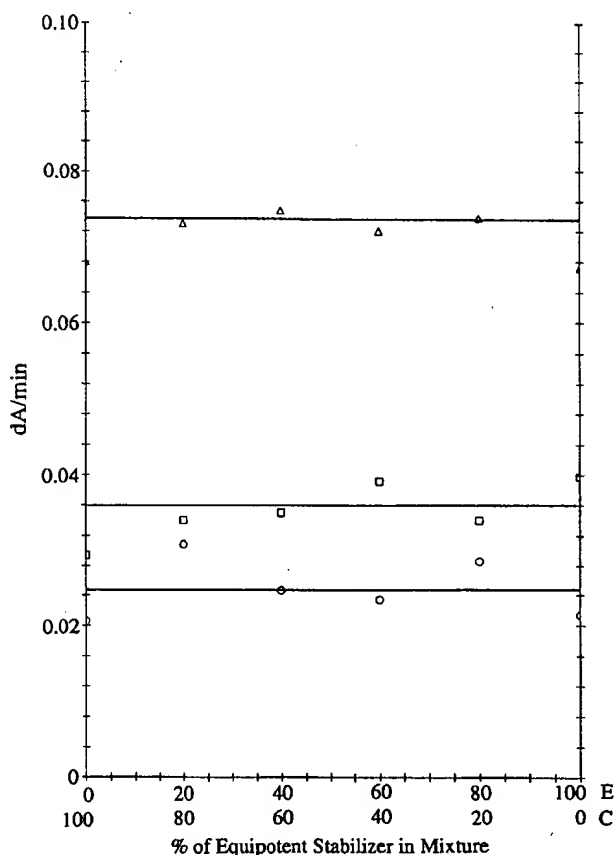


Fig. 8. Effect of varied enoxaparin (E) and sodium citrate (C) fractions of a priori equipotent combinations at various potency levels on the aggregation rate of RPR 114849 at pH 7.4 and 50°C. The 100% stabilizer concentrations (E in  $\mu\text{g/ml}$  and C in  $\mu\text{g/ml}$ ) are as follows: 3.7 E, 1.2 C (O); 2.5 E, 0.8 C (□); 0.9 E, 0.3 C (Δ).

### 3.10. Effect of gelling and microencapsulating agents

Increasing solution viscosity with a water soluble polymer, hydroxypropyl methyl cellulose (HPMC), slightly reduces the aggregate formation rate probably by hindering the intermolecular interaction of the denatured protein molecules (Table 1). Hydroxypropyl cyclodextrin (HPCD) has been cited for its ability to reduce aggregation of selected proteins (Brewster et al., 1989, 1991). But it was found to be only marginally effective (Table 1).

## 4. Conclusions

This study was designed to assess empirically the ability of potential stabilizers against heat-induced aggregation of the protein using a rapid screening procedure. Although some of the general protein stabilizers such as polyols sugars and amino acids were found to have some beneficial effect in stabilizing the protein, the concentration required is too much to be considered for practical use. Only polymeric anions can stabilize the protein to a greater extent. The trend determined by this technique should be verified by real-time stability studies under normal storage conditions. In the selection of stabilizers, many factors should be considered such as toxicity and anticoagulant activity in addition to their stabilizing ability.

## References

- Arakawa, T., Kita, Y., Carpenter, J.F., 1991. Protein-solvent interactions in pharmaceutical formulations. *Pharm. Res.* 8, 285–291.
- Brewster, M.E., Hora, M.S., Simpkins, J.W., Bodor, N., 1989. The potential use of cyclodextrins in parenteral formulations. *J. Parent. Sci. Technol.* 43, 231–240.
- Brewster, M.E., Hora, M.S., Simpkins, J.W., Bodor, N., 1991. Use of 2-hydroxypropyl- $\beta$ -cyclodextrin as a solubilizing and stabilizing excipient for protein drugs. *Pharm. Res.* 8, 792–795.
- Burgess, W.H., Maciag, T., 1989. The heparin-binding (fibroblast) growth factor family of proteins. *Annu. Rev. Biochem.* 58, 575–606.

- Chawla, A.S., Hinberg, I., Blais, P., Johnson, D., 1985. Aggregation of insulin containing surfactants, in contact with different materials. *Diabetes* 34, 420–424.
- Chen, T., 1992. Formulation concerns of protein drugs. *Drug Dev. Ind. Pharm.* 18, 1311–1354.
- Chen, B., Arakawa, T., 1996. Stabilization of recombinant human keratinocyte growth factor by osmolytes and salts. *J. Pharm. Sci.* 85, 419–422.
- Chen, B., Arakawa, T., Hsu, E., Narhi, L.O., Tressel, T.J., Chien, S.L., 1994a. Strategies to suppress aggregation of recombinant keratinocyte growth factor during liquid formulation development. *J. Pharm. Sci.* 83, 1657–1661.
- Chen, B., Arakawa, T., Morris, C.F., Kenney, W.C., Wells, C.M., Pitt, C.G., 1994b. Aggregation pathway of recombinant human keratinocyte growth factor and its stabilization. *Pharm. Res.* 11, 1581–1587.
- Cleland, J.L., Powell, M.F., Shire, S.J., 1993. The development of stable protein formulation: A close look at protein aggregation, deamidation, and oxidation. *Crit. Rev. Ther. Drug Carrier Syst.* 10, 307–377.
- Kajio, T., Kawahara, K., Kato, K., 1992. Stabilization of basic fibroblast growth factor with dextran sulfate. *FEBS Lett.* 306, 243–246.
- Lobb, R.R., Harper, J.W., Fett, J.W., 1986. Purification of heparin-binding growth factors. *Anal. Biochem.* 154, 1–14.
- Loughheed, W.D., Albisser, A.M., Martindale, H.M., Chow, J.C., Clement, J.R., 1983. Physical stability of insulin formulations. *Diabetes* 32, 424–432.
- Manning, M.C., Patel, K., Borchardt, R.T., 1989. Stability of protein pharmaceuticals. *Pharm. Res.* 6, 903–918.
- Shahrokh, Z., Eberlin, G., Buckley, D., Paranandi, M.V., Aswad, D.W., Stratton, P., Mischak, R., Wang, Y.J., 1994. Major degradation products of basic fibroblast growth factor: Detection of succinimide and iso-aspartate in place of aspartate. *Pharm. Res.* 11, 936–944.
- Timasheff, S.N., 1992. Solvent effects on protein stability. *Curr. Opin. Struct. Biol.* 2, 35–39.
- Tsai, P.K., Volkin, D.B., Dabora, J.M., Thompson, K.C., Bruner, M.W., Gress, J.O., Matuszewska, B., Keogan, M., Bondi, J.V., Middaugh, C.R., 1993. Formulation design of acidic fibroblast growth factor. *Pharm. Res.* 10, 649–659.
- Vemuri, S., Beylin, I., Sluzky, V., Stratton, P., Eberlein, G., Wang, J., 1994. The stability of bFGF against thermal denaturation. *J. Pharm. Pharmacol.* 46, 481–486.
- Volkin, D.B., Tsai, P.K., Dabora, J.M., Gress, J.O., Burke, C.J., Linhardt, R.J., Middaugh, C.R., 1993. Physical stabilization of acidic fibroblast growth factor by polyanions. *Arch. Biochem. Biophys.* 300, 30–41.
- Volkin, D.V., Verticelli, A.M., Bruner, M.W., Marfia, K.E., Tsai, P.K., Sardana, M.K., Middaugh, C.R., 1995. Deamidation of polyanion-stabilized acidic fibroblast growth factor. *J. Pharm. Sci.* 84, 7–11.
- Wang, Y.C.J., Hanson, M.A., 1988. Parenteral formulations of proteins and peptides: Stability and stabilizers. *J. Parent. Sci. Technol.* 42, S4–S24.